

Labeling Wild *D. discoideum* cells with Cell Tracker  
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Adapted from protocol of Natalie Fonville

## OBTAINING SOURCE PLATES

1) Grow *D. discoideum* cells for approximately 40-43 hours on SM plates at three different concentrations of spores. The following concentrations are good:  $10^5$  spores/ plate,  $3 \times 10^5$  spores/ plate, and  $10^6$  spores/ plate. After approximately 40 hours of growth the plate inoculated with  $3 \times 10^5$  spores will typically be ready for harvesting (i.e. loaded with cells, though not aggregating). By plating spores at three different densities you ensure that you will have at least one plate that is ready for harvesting after 40 hours of growth. The timing of growth is similar for *D. purpureum*, though when plating out at the spore densities described above it would be good to put the spores down about 37-40 hours before you want to harvest the cells.

When inoculating these plates with spores I recommend doing so by diluting such that 100 $\mu$ l of spore suspension contains the number of spores that you want (e.g. for a  $10^5$  spore plate you would want your suspension to be concentrated at  $10^5$  spores/100 $\mu$ l or more simply  $10^6$  spores/mL). Then simply deliver 100 $\mu$ l of this spore suspension to the plate along with 200 $\mu$ l of relatively fresh KA liquid culture.

Then spread using an ethanol flamed spreader. When flaming your spreader do not allow the ethanol to burn away in the flame but rather ignite the spreader with the flame and then move it out of the flame and allow the ethanol to burn away there. Also make sure to test the spreader against the agar surface to make sure that it is not too hot when it contacts your spores. When spreading the plate be thorough, making sure to cover the entire agar surfaces—take extra care to cover the periphery of the plate.

After spreading the plate let it dry for approximate 7 minutes with the lid ajar near the flame. Afterward reposition the lid and allow the plate to grow.

2) Before harvesting cells from the plates, prepare everything you will need for the protocol. See the page at the end of this document for a list of what you will need.

3) After approximately 40 hours of growth, decide which plate you want to use. You want to use plates that have the most cells on them but that have not yet started to initiate multicellular development. Examine the plates for loose streaming or later stages of development. If there are no signs of development (early or otherwise) you may consider using the cells on the plate for an experiment.

You can gauge whether or not there are cells on the plate by several things. First, the texture of the film on the plate will be subtly rippled. Second, if there are cells you should be able to see a brownish tinge on the plate when you hold it up to the light. Also cells have a much fresher smell than do bacteria.

4) Use an EtOH sterilized policeman to scrape the film off of each of your plates. Transfer the plate film to a 50mL Falcon tube that has approximately 15mL of KK2 in it. Do this for each of your clones.

Then gently shake the Falcon tubes until the sludge that you scraped off the plates has been completely suspended in the KK2. It may help to add approximately 15 mL KK2 more to the tube after you have shaken it a bit. Gently shake until the sludge has been completely suspended. When shaking, try to avoid making foam. Once suspended and you can see no more clumps, fill the Falcon tube almost to the top with KK2

5) Centrifuge the tubes at 1000 rpm (210 rcf) in the IEC centrifuge for 3 minutes, 3 times. The point of this is to remove the bacteria. With each wash you will see that the supernatant becomes clearer. After the third spin the supernatant should be rather clear. If it is not then you may consider doing a fourth spin, though this is not likely to be needed.

After each spin pour the supernatant out of the tube and then tap the bottom of the tube with your finger to break the pellet. After the first two spins add about 15-20 mL of KK2 and gently shake or tap to suspend the pellet. Then top off the tube with KK2 and spin again.

After the third and final spin pour off the supernatant and tap to break the pellet as usual, but add a smaller volume of KK2 so that it is not concentrated at less than  $1 \times 10^7$  cell/mL, but rather has a concentration of between approximately  $5 \times 10^7$  to  $7 \times 10^7$  cells/mL. With experience you will be able to eye-ball approximate this fairly well. Generally it takes between 3 to 12 mL of KK2 to suspend a pellet coming from one plate at these approximate concentrations.

7) Make a 1:20 dilution of each cell suspension. I usually do this by adding 50 $\mu$ l of the cell suspension to 950 $\mu$ l of KK2.

8) Place the Falcon tubes on the shaker in the new lab at a speed of approximately 40.

9) Count 2 aliquots of the 1:20 dilutions of your cells suspensions. Use these counts to estimate the concentration of your cell suspension.

10) Dilute your cell suspensions to a concentration of  $10^7$  cells/ mL. You will need to make enough of this suspension to cover the needs of your experiment.

For example, if you are planning on labeling  $1.4 \times 10^7$  cells then I recommend making approximately 5 mL of this  $10^7$  cells/ mL suspension (the following steps will explain this more fully).

11) Aliquot this  $10^7$  cells/ mL suspension into 2.0 mL centrifuge tubes. The size of your aliquot will depend on the needs of your experiment.

For example, if you are planning on labeling  $1.4 \times 10^7$  cells then you will need 1.4 mL in each of the centrifuge tubes. You will need at least two centrifuge tubes for each of your clones; however I recommend prepping three: 1 for CMF labeled cells and 2 for unlabeled cells. I recommend

doing two tubes for the unlabeled clones, so that you are less likely to have your experiment bust do to a lack of unlabeled cells, which (in comparison to the labeled cells) are not costly.

12) In a dim room add 1  $\mu\text{l}$  of 10mM Cell Tracker for every 200  $\mu\text{l}$  of  $10^7$  cells/mL suspension to the tube. Whenever working with Cell Tracker you should be in a dimly lit room.

To make 10mM Green Cell Tracker add 10.7  $\mu\text{l}$  of DMSO to a 50  $\mu\text{g}$  tube of the Green Cell Tracker. Pipet up and down several times to mix and ensure that you can recover the entire 10.7  $\mu\text{l}$ . Make 2 tubes at a time as you will need more than one for a single trial. Wrap the tubes in aluminum foil and place in a drawer.

For example, if you are have 1.4 mL of cell suspension in the tube you will need to add 7  $\mu\text{l}$  of 10 mM Cell Tracker to the tube.

13) Wrap all the tubes in aluminum foil and place on the platform shaker in the microscope room for 20 minutes.

14) Centrifuge the tubes at 1800 rpm (approximately 350 rcf) for 5 minutes.

15) Rotate each of the tubes so that the pellet is on the bottom of the tube while you work through steps 16 and 17. This will help maintain the integrity of the pellet and help prevent having cells slide out of your pellet do to gravity.

16) Use a micropipette to remove all of the supernatant from each of the tubes. Take extreme care when doing this to avoid cell loss. Draw the supernatant off slowly and smoothly. Never push the pipette plunger back in while removing the supernatant.

17) Flick the tubes so that the pellet breaks and then add 1 mL of KK2 to each of the tubes. Confirm that the pellet has resuspended.

18) Repeat steps 14-17.

19) After resuspending the pellet after the second spin wrap the tubes in aluminum foil and shake as in step 13 for another 20 minutes. This will give the cells an opportunity to efflux the excess dye.

20) Wash the excess CMF away by centrifuging and resuspending as in steps 14-17 three additional times. After the third spin do not add 1mL of KK2 but rather add approximately 150  $\mu\text{l}$  of PDF.

21) If you did multiple tubes of unlabeled cells for each clone (recommended above), pool the unlabeled cells together into one of the tubes.

22) Make a 1:10 dilution of these cells suspensions. I usually do this by adding 20  $\mu\text{l}$  of the cell suspension to 180  $\mu\text{l}$  of PDF.

23) Count two aliquots from each of these dilutions so that you can estimate the concentration of each cell type (i.e. Clone 1 labeled, Clone 2 labeled, Clone 1 unlabeled, Clone 2 unlabeled).

24) Dilute each of your suspensions to a fixed concentration.

I recommend  $4 \times 10^7$  cells/mL. At this concentration 50 $\mu$ l of suspension will contain  $2 \times 10^6$  cells. For a given experiment you will want to standardize both the volume and the number of cells.

25) Prepare chimeras by adding together equal volumes of the suspensions that you made in step 24. For a fully controlled experiment you will want to make the following mixes:

- Clone 1 (labeled) with Clone 2 (unlabeled)
- Clone 2 (labeled) with Clone 1 (unlabeled)
- Clone 1 (labeled) with Clone 1 (unlabeled)
- Clone 2 (labeled) with Clone 2 (unlabeled).

26) Deliver 50 $\mu$ l of the mixes from step 25 to the center of a prepared Nitrocellulose filter. When doing this start in the center of the filter and as you slowly eject the aliquot spiral outward from the center. Try to deliver the aliquot to a small an area as possible. You want to deliver the aliquot over a fairly standardized area across treatments.

In addition to the mixes you should also plate out on filters the following as controls:

- Clone 1 (labeled)
- Clone 2 (labeled)
- Clone 1 (unlabeled)
- Clone 2 (unlabeled)

To prepare nitrocellulose filters, first place a Pall filter pad in a small Petri dish. Add 1 mL of PDF to the Pall filter pad and then place a nitrocellulose filter on top of the moistened filter pad. Prior to use the nitrocellulose filter needs to be soaking in water.

The nitrocellulose filters can be re-used. After you have developed cells on a filter, transfer the filter to soak in 10mM EDTA. Then simply pour boiling water over the filters three times before you intend to use them again. Also when new, before use the nitrocellulose filters need to have been soaked overnight in 10mM EDTA to remove ions that seem to affect the Dicty cells. Before use replace the 10mM EDTA with deionized water since EDTA is known to kill cells. As the filters have been changed somewhat by the manufacturer, and dicty does not seem to like the new ones quite as much, please be careful with the filters and try to avoid puncturing or tearing them.

27) After delivering the cells to the nitrocellulose filter, add 1 mL of PDF to the Petri dish, ensuring that it is absorbed by the filter pad and does not run over the top of the filter. This excess PDF prevents the nitrocellulose from dying out.

28) Place the filters in a Tupperware box that contains moist paper towels (to further aid in preventing the nitrocellulose filters from drying out).

29) Place the box in the dark and allow the cells to develop. Because of the high salt concentrations in the PDF the cells will commence development rather quickly. You can expect there to be fruiting bodies within about 12 hours.

30) Make a 1:10 dilution of all tubes by adding 20  $\mu$ l to 180  $\mu$ l PDF.

31) Count 2 aliquots of each dilution for both the density and proportion glowing. Start with the chimeras as cross-labeling can occur, be sure to note any faint glowing as this could be an indication of cross-labeling. Count up to 500 total cells when counting the proportion glowing and try to avoid taking more than 5 pictures per aliquot. Switch aliquots once you reach about 250 total cells counted.

## DATA COLLECTION

### Total plate

1) Prepare a 1.5 ml tube for each plate that you have with 200  $\mu$ l of KK2+10mM EDTA for those plates containing  $2 \times 10^6$  cells and 1000  $\mu$ l for plates containing  $10^7$  cells plus an extra tube containing about 700  $\mu$ l to cool the loop in.

2) Flame a loop and cool in the extra tube of KK2+10mM EDTA. Leave the film of liquid that fills the loop.

3) Browse the tops of the fruiting bodies so that the sori pop into the liquid in the loop. Try to avoid knocking any over and do not touch the filter with the loop at this point as you will lose spores onto the filter. I find it helpful to turn off the overhead lights and turn on the hood light back in the microscope room as the light coming in from the side aids in picking out the fruiting bodies,

4) Swirl the loop in the appropriate tube and this time break the film of liquid inside the loop.

5) Use the loop to “mow” the fruiting bodies while trying to avoid knocking them over. You want them to stick to the loop in order to pick them up. I recommend doing just one straight line across the aggregation of fruiting bodies and then washing them off in the appropriate tube before beginning again. Repeat until all of the possible fruiting bodies have been scraped off the filter.

6) Do the same for all of the plates.

7) Start counting with the chimeras of glowing and non-glowing cells as you run the risk of losing some glowing if you have to stop for a period of time. Count 2 aliquots of each tube to figure out the density of spores as well as the proportion of cells you put down.

8) For the chimeras, once you count the density you then need to count the proportion of spores that glow. Count to a total of 500 spores and try to avoid taking more than 5 pictures per aliquot. Switch aliquots once you reach around 250 total spores counted. Compare this percentage glowing to the initial percentage by subtracting the proportion of spores glowing from the proportion of cells glowing. For the 100% labeled and unlabeled tubes just ensure that all (or none) of the spores are glowing, you don't need to count unless you feel that not all of the spores are glowing. Unless more than 1 or 2 spores are glowing in the unlabeled (which probably means the hemocytometer or cover slip wasn't cleaned well enough), you do not need to count these or any of the controls (which all come from the non-labeled stock of cells) for glowing.

#### Single sori

- 1) Place 7-10  $\mu$ l KK2+10mM EDTA on a plain glass slide (I usually do 2 per slide).
- 2) Using the fine point, #5 forceps carefully pick up a single fruiting body somewhere on the stalk and place it in the drop of KK2+10mM EDTA.

For species like *purpureum*, picking up an entire single fruiting body with the forceps is impossible. In this case, use the tip of one side of the forceps to pop the sorus and then place it in the drop of KK2+10mM EDTA.

- 3) Use the tip of the forceps to break up the sorus and spread the spores around. You can be a little violent at this point because the spores are extremely hard to count if they are in large aggregations.
- 4) Place a normal cover slip (not the fancy one) on to the drop by placing the edge of the cover slip along one edge of the drop of liquid and carefully lowering it. In the case of a popped sorus, the spores will normally be in the area of the edge of the cover slip that you put down first.
- 5) Count the proportion glowing, ensuring that you take pictures from a variety of places within the slide not all just in the same area.

#### 1% labeling

## Prep List for Cell Tracker Trial

1L of KK2

Cell Tracker in DMSO

1\_1 for every 200\_1 of labeled clone

Falcon Tubes for washing

Falcon Tubes for dilution to  $10^7$  cells/ mL

Count Dilution Tubes

1:20 (950\_1 KK2)

2.0mL Centrifuge Tubes

1 for every 1 mL of cell class

A L (labeled)

B L

A U (unlabeled)

B U

PDF tubes/ PDF dilution (1:10) tubes (180\_1 PDF)

1 of each for each cell class

1.5mL Mix tubes/ 1:10 Dilution Tubes for these

A + B 50/50 glow/no glow

B + A 50/50 glow/no glow

A + A 50/50 glow/no glow

B + B 50/50 glow/no glow

A 100% glow

B 100% glow

A 100% no glow

B 100% no glow

A(1% CMF) (if examining slug patterning)

B(1% CMF) “

A(1%) + B) “

B(1%) + A) “

4 agar starving plates (if examining slug patterning)

NC Filters + Development Box

Pall Filters—prep'ed with 1mL of PDF

NC Filter (boil washed 3 times)

Wet Towels in Box